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Arsenic inorganic compounds cause oxidative stress mediated by the transcription factor *PHO4* in *Candida albicans*

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ABSTRACT

Arsenic is a toxic metalloid widespread in nature. Recently, it has been demonstrated a main role of the transcription factor Pho4 in the acquisition of tolerance to arsenic-derived compounds, arsenite and arsenate in *Candida albicans*. Here, the effect of these compounds on this pathogenic yeast has been analyzed. In wild type cells, both arsenite and arsenate induced a marked increase in the endogenous production of Reactive Oxygen Species (ROS), together with the accumulation of intracellular trehalose and the activation of catalase, suggesting their role as generators of oxidative stress in this yeast. However, a *pho4* null mutant showed a minor increase of intracellular ROS and a different kinetics of catalase activation upon exposure to arsenite and arsenate. Interestingly, the enzymatic activity of glutathione reductase and superoxide dismutase were exclusively triggered by arsenite but not by arsenate. *pho4* mutant cells were also found to be sensitive to azide but significantly resistant to arsenate through a process dependent on an active electron transport chain and the alternative oxidase system. Therefore, arsenic-derived compounds induce a strong antioxidant response in *C. albicans* via different mechanisms.

1. Introduction

Arsenic is an extensively distributed metalloid in the environment, either from natural or anthropogenic sources, and is considered an important health problem in many countries and industries. It has usually been associated with several life-threatening diseases such as cardiovascular and peripheral vascular diseases, neurologic disorders and diabetes (Tchounwou et al., 2003 Watanabe and Hirano, 2013; Sun et al., 2014). Also, different tumors might be developed by prolonged arsenic exposure (Tapio and Grosche, 2006). The main route to arsenic exposure in occupational cases is inhalation. That is the case of different industry workers. Ingestion of contaminated drinking water or food is the predominant source of environmental arsenic exposure in contaminated places. The trivalent (As (III)) and pentavalent (As (V)) forms of arsenic are the most common inorganic derivatives present in nature. As (III) is more toxic than the pentavalent compound and is the predominant in anaerobic niches. As (III) enters the cells through aquaglyceroporins that function as active glycerol transporters, while As (V) uptake uses the phosphate transporters due to their structural homology. Once inside the cell, As (V) is reduced to As (III) by the arsenate reductases (Mukhopadhyay and Rosen, 2002).

As (V) can also be reduced non-enzymatically by means of glutathione (GSH) and other thiols. As (V) reduction to As (III) is required to take out this compound through aquaglyceroporins. In humans, several mechanisms have been proposed to explain the carcinogenicity of arsenic which include oxidative stress generation, perturbation of MAPK signal transduction, induction of apoptosis, inhibition of DNA repairing processes, global changes in DNA methylation and histone modifications (revised by (Tapio and Grosche, 2006; Rossman and Klein, 2011). Nevertheless, in the animal models so far tested, arsenic is unable to induce tumors, making difficult the study of arsenic toxicity. Thus, simple eukaryotic organisms may be a powerful tool in order to elucidate the complex mechanisms involved in human diseases (Dilda et al., 2008). In this way, previous studies in the model yeast *Saccharomyces cerevisiae* suggest that arsenite (As (III)) acts as a direct inducer of DNA breaks throughout the cell cycle (Litwin et al., 2013), and also provokes the oxidation of nitrogen bases and single strand breaks. These processes have also been observed in the fission yeast

Abbreviations: As (III), arsenite; As (V), arsenate; ROS, reactive oxygen species; GR, glutathione reductase; SOD, superoxide dismutases

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Schizosacharomyces pombe, suggesting that this mechanism of action must be conserved in other organisms (Litwin et al., 2013). Arsenic-derived compounds enter *S. cerevisiae* cells by a similar way to that unraveled in mammalian cells. Thus, As (V) enters yeast cells through the specific phosphate transporters: Pho84 and Pho89 (two high-affinity permeases) or Pho87 and Pho90 (two low-affinity permeases) (reviewed by Wysocki and Tamas, 2010). Once inside the cells, the arsenate reductase Arr2 reduces arsenate to arsenite. On the other hand, As (III) is up taken by yeast cells through the aquaglyceroporin Fps1 (Wysocki et al., 2001) and other permeases (Liu et al., 2004). Fps1 acts as a bidirectional channel allowing the efflux of As (III) (Maciaszczyk-Dziubinska et al., 2010). Notably, As (III) can also be transported into the vacuole as a glutathione-conjugated substrate by the Ycf1 ABC-transporter (Ghosh et al., 1999). Vujcic and co-workers (Vujcic et al., 2007) have analyzed in *S. cerevisiae* the effect of arsenic on mitochondrial activity/function, since consistent work has concluded that mitochondria are an important target for arsenic-induced cancer and cell death. Within the mitochondria, arsenic seems to uncouple the oxidative phosphorylation, because ATP synthase incorporates As (V) instead of phosphate to the ADP molecule. A screening using mutants deficient in mitochondria biogenesis, allowed the identification of several mutants sensitive to either As (III) or As (V) or both (Vujcic et al., 2007). The corresponding genes identified are involved in different mitochondrial activities, such as nucleic acid metabolism, oxidative phosphorylation, protein biosynthesis and/or vacuolar acidification.

Apart from the poisonous effect on human health, the ingestion of arsenic inorganic compounds influence the intestinal microbiota (Pinyayev et al., 2011) in a reciprocal exchange that generates intermediate compounds, more or less toxic than arsenic itself (Van de Wiele et al., 2010; Lu et al., 2014). The arsenic-induced alteration of the microbiota and its metabolism could explain the origin of human diseases associated to the arsenic ingestion. Indeed, the new findings highlight the key relevance of microbiota. *Candida albicans* is a polymorphic fungus that forms part of the normal microbiota of skin and mucosa including gut. Recently, a screening has identified five transcription factors (TFs) involved in the tolerance to arsenate, whose absence gives rise to arsenate-sensitive mutants (Urrialde et al., 2015). Remarkably, the bHLH transcription factor Pho4, belonging to the myc-family was identified. Pho4 was previously reported to be required for growing on phosphate-deficient medium and for virulence in a phosphate-depleted virulence model in *Caenorhabditis elegans* (Romanowski et al., 2012). The lack of Pho4 renders cells sensitive to both As (III) and As (V), although Pho4 expression was induced by As (V) addition, but not in the presence of As (III), suggesting that the two compounds trigger different responses in *C. albicans.* Later on, the role of Pho4 in protection against cation (Na^+) exposure (Ikeh et al., 2016) as well as in resistance to oxidative and osmotic stress has been reported (Urrialde et al., 2016). A *pho4* mutant displays reduced adhesion to murine gut mucosa and becomes less competitive than the wild type strain in either *in vitro* or *in vivo* assays and therefore, the metabolic role of Pho4 seems to be crucial to preserve fitness attributes. In the present work, the role of this transcription factor in the control of the oxidative metabolism has been explored as well as the effect of arsenic derived compounds on the oxidative stress defenses.

2. Materials and methods

2.1. Strains and growth conditions

The strains used in the present work were described previously (Urrialde et al., 2015). The cells were grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose), YPG (1% yeast extract, 2% peptone, 2% glycerol), SD (2% glucose, 0.67% yeast nitrogen base plus amino acids) or SG (2% glycerol, 0.67% yeast nitrogen base plus amino acids). Media were solidified with 2% agar. Growth in liquid medium was measured by cell density at OD_{600} or by direct cell count in a TC-20 cell counter (BioRad). As (V) or arsenate refers to Na_2HAsO_4 (disodium hydrogen arsenate or sodium arsenate dibasic). As (III) refers to $NaAsO_2$ or sodium arsenite. SHAM or salicylhydroxamic acid (Aldrich) was used to inhibit alternative oxidase activity while 2,4-DNP or 2,4-dinitrophenol was used for uncoupling oxidative phosphorylation.

Sensitivity to different compounds (sodium azide, SHAM, etc.) was tested on solid medium. 1/10 serially diluted cell suspensions were spotted to examine the growth of the different strains. Plates were incubated overnight at 37 ˚C in different conditions except in the case of anaerobiosis that were incubated 48 h before taking the pictures.

The influence of environmental conditions was analyzed in aerobic conditions named normoxia (around 21% O_2), normoxia plus 5% CO_2 or anaerobic atmosphere. Normoxia plus 5% CO_2 was achieved using an incubator designed for cell culture. This incubator was programmed at 37 ˚C, 80% humidity and 5% CO_2 in the presence of atmospheric O_2. Anaerobiosis was reached using an anaerobic chamber and the commercial system GENBox anaer (BioMerieux).

2.2. Preparation of cell-free extracts

After exposure to different stresses, samples from the cultures were harvested and resuspended at known densities (10–15 mg/ml, wet weight) in the extraction buffer, 100 mM 4-morpholine-ethanesulfonic acid (MES) pH 6.0, containing 5 mM cysteine and 0.1 mM phenyl methyl sulphonyl fluoride (PMSF). The cellular suspensions were transferred into small pre-cooled tubes (1.0 cm-diameter) with 1.5 g Ballotini glass beads (0.45 mm diameter). Cells were broken by vigorously vibrating the tubes in a vortex mixer. The tubes were quickly cooled in ice. The crude extract was then centrifuged at 10,000*g* for 5 min and the pellet was resuspended in the same buffer at the initial density.

2.3. Enzymatic assays

Catalase activity was determined at 240 nm by monitoring the removal of H_2O_2, as described elsewhere (Gonzalez-Parraga et al., 2003). Glutathione reductase (GR) activity was assayed by measuring the GSSG-dependent oxidation of NADPH as described in (Gonzalez-Parraga et al., 2003). Measurements of superoxide dismutase (SOD) were made spectrophotochemically by the ferricytochrome C method using xanthine/xanthine oxidase as the source of $O_2.-$ radicals (Gonzalez-Parraga et al., 2003). Data of enzymatic activity were normalized in relation to a control measurement (100%). Fumarase activity was analyzed at 240 nm following the transformation of malate to fumarate according to Walk and Hock (1977a,b).

Measurements of superoxide dismutase were carried out using the SOD Assay Kit (Sigma Aldrich), according to the manufacturer's instructions. SOD catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen. SOD activity was monitored spectrophotochemically by the ferricytochrome c method using xanthine/xanthine oxidase as the source of O_2^- radicals (McCord and Fridovich, 1969). One unit of activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome c reduction rate measured by a colometric method (λ = 440 nm) using a spectrophotometer (Fluostar Omega. BMG Labtech). Relative enzymatic activity was calculated as the ratio of treated cells *versus* untreated cell at each time point.

2.4. Cytometric analysis

Intracellular ROS formation using dihydrofluorescein diacetate (DHF) and mitochondrial membrane potential with rhodamine 123 (R123) were quantified by flow cytometry. Briefly, overnight cultures from the different *C. albicans* strains were refreshed in new and warm medium, either YPD or SG as indicated in the Figure and incubated till